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EXPERIMENTAL ARTICLES

# Diversity of Diazotrophs in the Sediments of Saline and Soda Lakes Analyzed with the Use of the *nifH* Gene as a Molecular Marker

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**Abstract**—Phylogenetic analysis of the *nifH* genes, encoding the Fe protein of the nitrogenase enzymatic complex, was carried out for pure cultures of anoxygenic phototrophic bacteria of diverse origin, as well as for heterotrophic alkaliphilic sulfate reducers isolated from saline and soda lakes. Topology of the nitrogenase tree correlated with that of the 16S rRNA gene tree to a considerable degree, which made it possible to use the *nifH* gene as a molecular marker for investigation of diazotrophic bacterial communities in sediments of hyper saline and soda lakes. Although diazotrophs were revealed in all environmental samples, their phylogenetic diversity was relatively low. Sulfate-reducing deltaproteobacteria and photo- and chemotrophic gammaproteobacteria were predominant in integrated samples. Analysis of the upper sediment layers revealed predominance of phototrophic diazotrophs of various phyla, including purple sulfur and nonsulfur proteobacteria, green nonsulfur bacteria, heliobacteria, and cyanobacteria. Some phylotypes could not be identified, probably indicating the presence of bacterial groups which have not yet been studied by conventional microbiological techniques.

*Keywords*: saline and soda lakes, nitrogen fixation, diazotrophs, *nifH* gene

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The prokaryotes capable of fixing molecular nitrogen (diazotrophs) are present in virtually all ecosystems, where they play the important role of providing bound nitrogen. However, their role in extreme habitats, inland hypersaline lakes in particular, remains insufficiently studied. It was shown that in saline lakes the nitrogen fixation (NF) process is driven by moderately halophilic cyanobacteria and by anaerobic bacteria, whose activity is suppressed by oxygen and light [1]. Study of the diversity of diazotrophs in the cyanobacterial mats of saline lakes showed that the composition of their population changes with the seasonal variations in salinity [2].

Soda lakes are a specific type of saline lakes, characterized by prevalence of sodium carbonates/bicarbonates, which determine a steadily high pH value (9.0–11.0) in these ecosystems. As a result, the conditions are unique, being extreme in terms of both salinity and alkalinity; they suppress eukaryotic life and lead to the development of natronophilic prokaryotic communities. Recent intensive studies of the prokaryotic communities of soda lakes revealed the main players of the biogeochemical cycles of carbon, nitrogen, and sulfur; all of the pure cultures that were isolated are those of haloalkaliphilic species, which form a prokaryotic structural and functional system [3–5]. In this system, microorganisms representing two types of metabolism are supposedly potentially capable of NF: microorganisms with phototrophic metabolism, such as haloalkaliphilic aerobic oxygenic cyanobacteria and anoxygenic purple and green nonsulfur bacteria (green sulfur bacteria of the *Chlorobi* phylum have not yet been found in soda lakes), and microorganisms with heterotrophic metabolism, such as haloalkaliphilic primary and secondary anaerobes (*Firmicutes*, spirochetes, *Deltaproteobacteria*, methanogenic archaea). However, the experimental evidence of NF in soda lakes is so far scarce. In particular, direct methods employing the $^{15}N$ isotope revealed NF in the littoral zones of soda lakes in California and Nevada (United States), and it was ascribed to haloalkaliphilic cyanobacteria, which are capable of actively driving the process at pH 10 and mineralization of up to 100 g/L [6, 7]. In spite of the evident presence of natronophilic cyanobacteria in soda lakes, their role in the nitrogen cycle, as well as the distribution of the NF capacity among them, remains virtually unstudied. As for the heterotrophic components of the ecosystem, recent measurements of the potential rate of NF in soda

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solonchaks and anaerobic sediments of soda lakes in Kulunda steppe (Altai, Russia) demonstrated the presence of an active diazotrophic population of natronophilic aerotolerant fermenters belonging to new taxa of the *Firmicutes* [8–10].

The nitrogenase enzymatic complex, which is the key one for the NF processes, consists of two subunits: the FeMo protein, encoded by the *nif*D and *nif*K genes, and the Fe protein, encoded by the *nifH*, the molecular marker most commonly used in studies of known diazotrophs and prokaryotes potentially capable of NF. The value of this marker is determined by its evolutionary conservativeness and by the good correlation of its phylogeny with the 16S rRNA gene phylogeny. In the last several years, NF capacity and/or presence in the genome of the *nifH* gene were found in a number of haloalkaliphilic isolates from soda lake sediments, such as *Clostridium alkalicellum* [11], *Geoalkalibacter ferrihydriticus* [12], *Thioalkalispira microaerophila* and *Alkalilimnicola halodurans* [13]; however, no detailed studies were conducted on the diazotrophy in representatives of these new taxa.

Molecular-ecological studies of saline and soda lakes with the use of key metabolic genes includes studies on the diversity of autotrophs [14–17], sulfur-oxidizing bacteria [18], sulfate-reducing bacteria [19], and methanogens [20]. A single study used the *nifH* gene as molecular marker, and it was conducted with samples from different layers of the Mono Lake (California) water column [21]. That work showed prevalence of clones of the *nifH* genes of sulfate-reducers that belonged to *Deltaproteobacteria* in the anaerobic portion of the water column and to *Gammaproteobacteria* in the aerobic portion. However, NF determinations in the water column produced negative results, probably indicating an inactive state of the diverse potentially diazotrophic population of the Mono Lake water column.

In the present work, *nifH* genes were used as molecular markers to evaluate the diversity of diazotrophic prokaryotes in surface and integral samples of sediments of soda and hyper saline lakes in Siberia and Egypt. Additionally, with the aim of database replenishment, we amplified, sequenced, and phylogenetically analyzed (with comparison to 16S rRNA gene phylogeny) *nifH* genes in several collection cultures of phototrophic purple bacteria of various origins, as well as in a number of strains belonging to new genera of deltaproteobacterial sulfate reducers isolated from soda lakes.

## MATERIALS AND METHODS

**The study subjects** were strains of purple phototrophic microorganisms of various origin from the collection of the Department of Microbiology, Moscow State University; a recent soda lake isolate, strain B8-1, identified as representing the species *Thiorhodospira sibirica* based on 16S rRNA gene analysis [17]; and a group of strains of natronophilic sulfate reducers belonging to recently described deltaproteobacterial taxa, isolated from soda lakes and maintained in the culture collection of the Institute of Microbiology, RAS (Table 1).

Integral samples and samples of surface layers of silty sediments of hypersaline chloride–sulfate and soda lakes of Kulunda Steppe (Altai Krai) and alkaline hypersaline Wadi El Natrun lakes (Libyan Desert, Egypt) were used in this work (Table 2). Sediment samples from small soda lakes were pooled in cases of close pH values and salinity (mostly moderate). The samples were stored at 4°C until use.

**DNA isolation** from pure cultures was carried out with the MoBio kit (Ultra Clean Microbial DNA Isolation kit; MoBio Laboratories) according to the manufacturer's recommendations.

The DNA isolation from sediments of soda lakes used the kit for isolation of total DNA from soils (MoBio Power Soil DNA Isolation kit; MoBio Laboratories). Approximately 10 g of sediment was washed with 1 M NaCl; the suspension was centrifuged at 3000 *g* for 2 min to precipitate sand and large silt particles, and the supernatant was transferred to another tube and centrifuged once more. To obtain colloidal fraction, two to three times repeated centrifugation was required, depending on the sediment type. From the colloid thus obtained, a 2-mL portion was taken and used for DNA isolation after adjustment of pH to 7.5 with 0.5 M HCl.

**Amplification of *nifH* genes on DNA from pure cultures and sediment samples** was performed using earlier designed primes according to the earlier described protocol [22]. The amplificates obtained were subjected to electrophoresis in 1.0% agarose gel stained with ethidium bromide (0.5 μg/mL). PCR fragments of the expected length were excised from the gel, purified using the Wizard PCR Preps kit (Promega, United States), and sequenced immediately (for pure cultures) or after cloning (for soda lake sediment samples).

**Cloning** was performed using the CloneJet PCR Cloning kit (Fermentas) including plasmid pJET1.2/blunt according to the manufacturer's protocol. From each clone library, 25–50 clones were chosen to be tested for the presence of the insert by PCR with specific primers, followed by electrophoresis of the products in 1.0% agarose gel stained with ethidium bromide (0.5 μg/mL). Clones containing the target insert were sequenced and the sequences were used for further analysis.

**Phylogenetic analysis.** The sequences were edited with the use of BioEdit [http://jwbrown.mbio.ncsu.edu/BioEdit/bioediTca.html]. Primary comparison of the de novo determined sequences with sequences available in the GenBank database was performed by using BLAST at the NCBI site [http://www.ncbi.nlm.nih.gov/blast]. For further comparative

**Table 1.** Pure cultures of diazotrophic microorganisms studied in this work

| Order | Species | Strain | Length of sequenced *nifH* gene fragment (bp) | Collection/Isolation source |
|---|---|---|---|---|
| Gammaproteobacteria | | | | |
| *Chromatiales* | *Thiorhodospira sibirica* | B8-1 | 393 | INMI RAS/soda lake Zun-Torei (Transbaikal region) |
| | *Thiocapsa* sp. | 1R | 399 | Department of Microbiology, MSU |
| Alphaproteobacteria | | | | |
| *Rhizobiales* | *Rhodoblastus acidophilus* | 5–6 | 447 | Department of Microbiology, MSU |
| | *Rhodoplanes elegans* | AS130$^T$ | 408 | |
| | *Rhodoplanes roseus* | 941$^T$ | 381 | |
| | *Rhodomicrobium vannielii* | K-1 | 408 | |
| *Rhodobacterales* | *Rhodovulum sulfidophillum* | Rp-6 | 447 | |
| | *Rhodovulum eurihalinum* | DSM 4868$^T$ | 390 | |
| | *Rhodovulum adriaticum* | DSM 2781$^T$ | 447 | |
| Betaproteobacteria | | | | |
| *Burkholderiales* | *Rubrivivax gelatinosus* | DSM 149 | 447 | Department of Microbiology, MSU |
| Deltaproteobacteria | | | | |
| *Desulfovibrionales* | *Desufonatronovibrio halophilus* | HTR 9 | 399 | INMI RAS/hypersaline lakes (Kulunda Steppe) |
| | | HTR 10 | 426 | |
| | *Desulfonatronum thioautotrophicum* | ASO4-1$^T$ | 414 | INMI RAS/soda lakes (Kulunda Steppe) |
| | *Desulfonatronum thiosulfatophilum* | ASO4-2$^T$ | 402 | |
| *Desulfobacterales* | *Desulfobotulus alkaliphilus* | ASO4-4$^T$ | 390 | INMI RAS/soda lakes (Kulunda Steppe) |
| | *Desulfobulbus alkaliphilus* | APS1$^T$ | 393 | INMI RAS/soda lakes (Kulunda Steppe) |

analysis, *nifH* gene sequences were retrieved from GenBank. Nucleotide sequences of the genes studied and amino acid sequences deduced were aligned with relevant sequences of the closest bacterial species with the use of CLUSTALX 2.0 [http://bips.u-strasbg.fr/fr/Documentation/ClustalX/]. Phylogenetic trees were constructed using the TREECONW [http://bioc-www.uia.ac.be/u/yvdp/treeconw.html] and MEGA 5.2 [http://www.megasoftware.net/] software packages. The statistical significance of the branching order was determined by bootstrap analysis of 1000 alternative trees and expressed in percent.

The Good's coverage index [23] for the samples was calculated by the formula $C = 1 - (n/N)$, where $n$ is the number of unique (single) sequences, and $N$ is the total number of analyzed sequences. The Chao1 species richness index was calculated using the EstimateS program [Version 8, R.K. Colwell, http://purl.oclc.org/estimates].

**Deposition of nucleotide sequences.** The nucleotide sequences of the *nifH* genes determined in this work were deposited in GenBank with accession numbers KF800047–KF800091.

## RESULTS AND DISCUSSION

**Detection and phylogenetic analysis of *nifH* genes from pure cultures of phototrophic and sulfate-reducing bacteria.** Most of phototrophic bacteria from different phyla are capable of fixing molecular nitrogen via the operation of enzymes of the nitrogenase complex. This capacity occurs not only to the photoautotrophs that employ the Calvin cycle (alpha-, beta-, and gammaproteobacteria, green nonsulfur bacteria, and some cyanobacteria) but also to autotrophic green sulfur

**Table 2.** Characterization of the habitats of the microbial communities and the clone libraries derived from them

| Sample | Year of sampling | Region | Lake | Lake type | Characteristics of the brine | | | Characteristics of sediment samples | Number of clones | Number of phylotypes | Diversity (phylotypes/clones) | Good's coverage index (%) | Chao1 (min, max); coverage (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | pH | salinity (g $L^{-1}$) | alkalinity (M) | | | | | | |
| 05-3 | 2005 | Kulunda Steppe (Altai, Russia) | Bitter Lake system (integrated samples from 3 lakes) | Hypersaline soda lakes | 10.2–10.4 | 30–130 | 0.2–1.2 | Integral (0–10 cm) | 57 | 10 | 0.17 | 94.7 | 10 (10, 18); 100 |
| 4 KL | 2009 | | Bitter-1 | | 10.2 | 300 | 4.70 | Superficial (0–1 cm) | 21 | 5 | 0.24 | 90.5 | 7 (5, 27); 75 |
| 6 KL | | | Tantar-5 | | 10.1 | 180 | 1.60 | | 59 | 5 | 0.08 | 97.0 | 5 (5, 13); 100 |
| 2 KL | | | Cock Salt lake | Hypersaline chloride–sulfate lakes | 7.7 | 300 | – | | 23 | 4 | 0.18 | 100 | 4 (4, 4); 100 |
| 14 KL | 2007 | | Burlinskoe | | 7.45 | 300 | – | Integral (0–10 cm) | 20 | 1 | 0.05 | 100 | 1 (1, 1); 100 |
| WN | 2001 | Wadi El Natrun | Integrated samples from 8 lakes | Alkaline hypersaline lake | 9.5–10.1 | 200–360 | 0.2–1.5 | | 20 | 4 | 0.20 | 95 | 4 (4, 4), 100 |

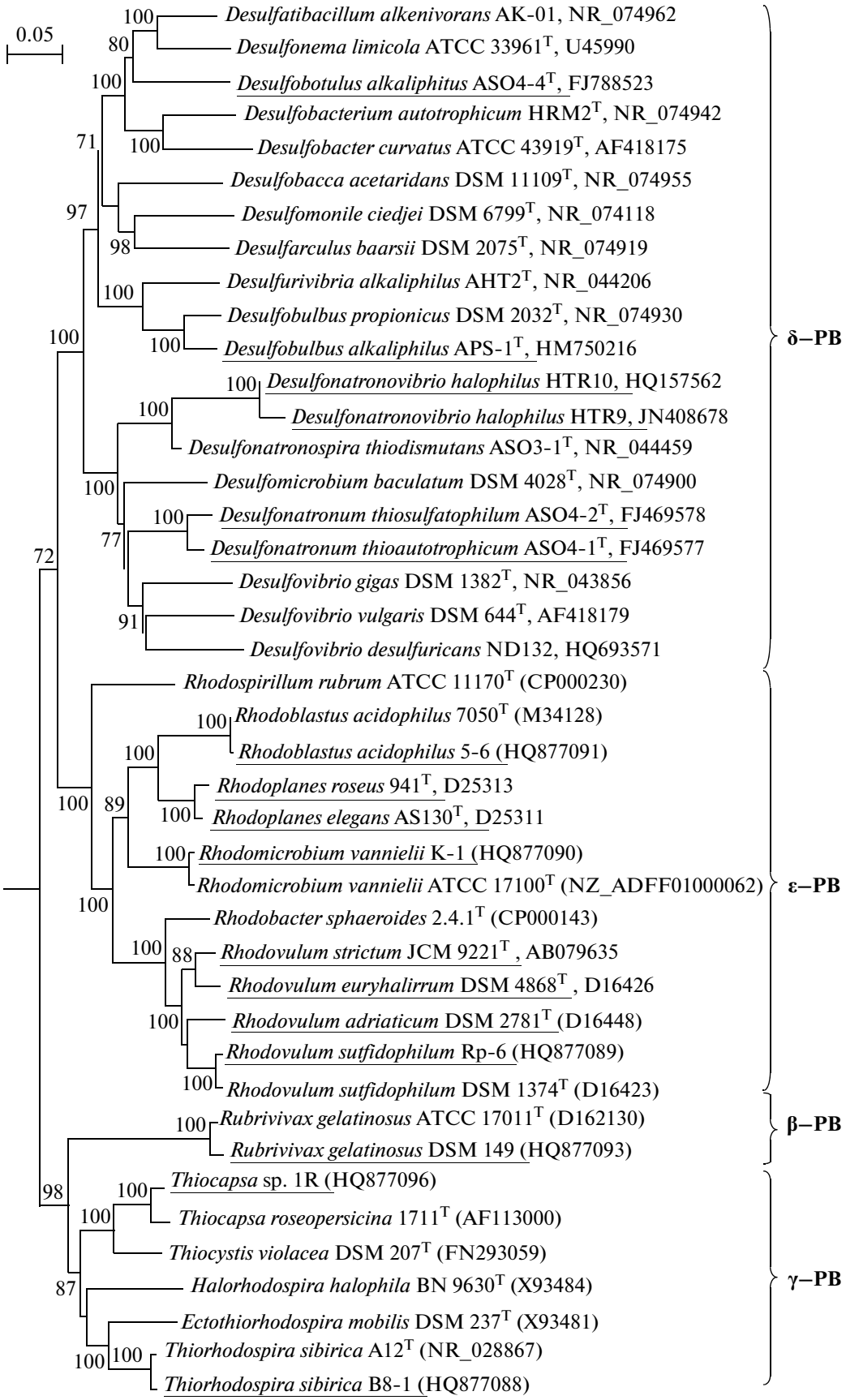
bacteria, which fix carbon dioxide via the reductive tricarboxylic acid cycle, and to gram-negative photoheterotrophic heliobacteria. It has been argued that the processes of photosynthesis and NF are interrelated, as evidenced by the structural similarity of the nitrogenase Fe protein, encoded by the *nifH* gene, and certain enzymes of chlorophyll biosynthesis [24]. NF is also common among sulfate-reducing deltaproteobacteria; however, genes of the nitrogenase complex have so far been revealed in the genomes of only two representatives of natronophilic sulfidogens: the sulfur reducer/disproportionator *Desulfurivibrio alkaliphilus* AHT2 (CP001940) and the sulfate reducer *Desul-fonatronospira thiodismutans* ASO3-1 (NZ_ACJN02000001). In our present work, we revealed and sequenced *nifH* genes in nine collection cultures of photoautotrophic alpha-, beta, and gammaproteobacteria of various origins, as well as in five strains of alkaliphilic sulfate-reducing deltaproteobacteria isolated from soda lakes (Table 1). PCR products of the expected length (about 450 bp) were obtained for all of the studied strains, and search in the GenBank database showed the affiliation of the de novo determined nucleotide sequences to the *nifH* gene family. These sequences were further used for comparative analysis, together with proteobacterial NifH sequences available from GenBank.

The topology of the constructed nitrogenase tree of proteobacteria correlated to a considerable extent with that of the 16S rRNA gene tree (Figs. 1, 2). Such a correlation was earlier noted for other bacterial groups [25] and is indicative of the coevolution of these functionally independent genes. In the nitrogenase tree (Fig. 2), the proteobacteria are distinctly separated into classes, and most of the newly studied strains occupy the same positions as in the 16S rRNA gene tree (Fig. 1). The only exception among purple phototrophic bacteria was the strain *Rhodomicrobium vannielii* K-1. In the genome sequence of the type strain of this species, two genes encoding Fe protein were revealed earlier: a commonly occurring gene (*nifH*) and an alternative gene (*anfH*) associated with the V-containing nitrogenase. Although the 16S rRNA gene sequences of the *Rm. vannielii* type strain and strain K-1 are nearly identical (99.3%), only the *anfH* alternative gene was revealed in the total DNA of strain K-1. In the cluster formed by the alternative genes, the relevant sequences of these strains were the closest, albeit the level of their divergence was higher (8.2% for the nucleotide sequence and 1.5% for the deduced amino acid sequence) that the divergence level of the 16S rRNA genes. For the ordinary *nifH* genes of the other strains studied, the degree of intraspecies divergence varied in a wider range (0.2–8.4% for nucleotide sequences and 0–3.3% for amino acid sequences) than the degree of divergence of 16S rRNA genes (0.5–1.5%). In general, the relative degree of conservation of *nifH* and 16S rRNA genes may be different at different taxonomic levels, and this causes local discrepancies between the topologies of phylogenetic trees [25]. In particular, in the trees constructed in the present work, examples of such discrepancies are the greater proximity of alpha- and betaproteobacteria in the nitrogenase tree compared to the 16S rRNA gene tree and different positions occupied by the new taxa of sulfate reducers within the deltaproteobacterial clusters. Nevertheless, species identification of diazotrophic strains based on *nifH* gene analysis proved to be quite reliable, and this allows this gene to be used as a molecular marker in molecular-ecological studies of natural prokaryotic communities.

**Detection and phylogenetic analysis of *nifH* genes in sediment samples from hypersaline and soda lakes**. Application of specific *nifH*-targeted primers allowed us to obtain relevant amplification products for all of the samples studied. The clone libraries that we constructed (20–59 clones for each sample) contained one to ten distinct NifH phylotypes (at a nucleotide sequence identity level within a particular phylotype of 97% or higher); the compositions of the libraries differed considerably between the samples. Our statistical analysis of the distribution of nucleotide sequences between phylotypes within the libraries showed that the analysis of the clones that we obtained provided for reliable determination of the diversity of sequence types (Table 2): Good's coverage index [23] was 90.5–100%, and, for most of the libraries, maximum coefficient of diversity coverage was achieved (the Chao1 index of 100%). Only in the 4KL library was this index as low as 75%, indicating insufficiency of the number of the analyzed sequences for making reliable conclusions about the NifH diversity in this sample. The phylogenetic tree of NifH phylotypes represented in the clone libraries is shown in Fig. 3.

During the analysis of clone libraries, it is usually assumed that the number of identical clones containing PCR-amplified marker genes is proportional to the initial concentration of the template DNA belonging to the respective member of the community. Therefore, the ratios of the particular clones obtained in such experiments should correlate with the initial ratios of the respective community members. This correlation is not quite direct and its strictness depends on the selectivity of the template DNA isolation and subsequent amplification and cloning. Nev-

**Fig. 1.** 16S rRNA gene-based phylogenetic tree showing the position of the studied strains of diazotrophs among proteobacteria. Underlined are strains for which *nifH* genes were sequenced in this work. The tree was constructed with the use of the Neighbor-Joining algorithm. Scale bar shows evolutionary distance corresponding to 5 substitutions per 100 nucleotides. Numerals at the nodes show the statistical significance of the branching order as determined by bootstrap analysis of 1000 alternative trees (only values higher than 70% are shown).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Desulfarculus baarsii DSM 2075T, NR_074919
Desulfurivibria alkaliphilus AHT2T, NR_044206
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Desulfonatronospira thiodismutans ASO3-1T, NR_044459
Desulfomicrobium baculatum DSM 4028T, NR_074900
Desulfonatronum thiosulfatophilum ASO4-2T, FJ469578
Desulfonatronum thioautotrophicum ASO4-1T, FJ469577
Desulfovibrio gigas DSM 1382T, NR_043856
Desulfovibrio vulgaris DSM 644T, AF418179
Desulfovibrio desulfuricans ND132, HQ693571
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Rhodospirillum rubrum ATCC 11170T (CP000230)
Rhodoblastus acidophilus 7050T (M34128)
Rhodoblastus acidophilus 5-6 (HQ877091)
Rhodoplanes roseus 941T, D25313
Rhodoplanes elegans AS130T, D25311
Rhodomicrobium vannielii K-1 (HQ877090)
Rhodomicrobium vannielii ATCC 17100T (NZ_ADFF01000062)
Rhodobacter sphaeroides 2.4.1T (CP000143)
Rhodovulum strictum JCM 9221T , AB079635
Rhodovulum euryhalirrum DSM 4868T , D16426
Rhodovulum adriaticum DSM 2781T (D16448)
Rhodovulum sutfidophilum Rp-6 (HQ877089)
Rhodovulum sutfidophilum DSM 1374T (D16423)
ε–PB
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β–PB
Thiocapsa sp. 1R (HQ877096)
Thiocapsa roseopersicina 1711T (AF113000)
Thiocystis violacea DSM 207T (FN293059)
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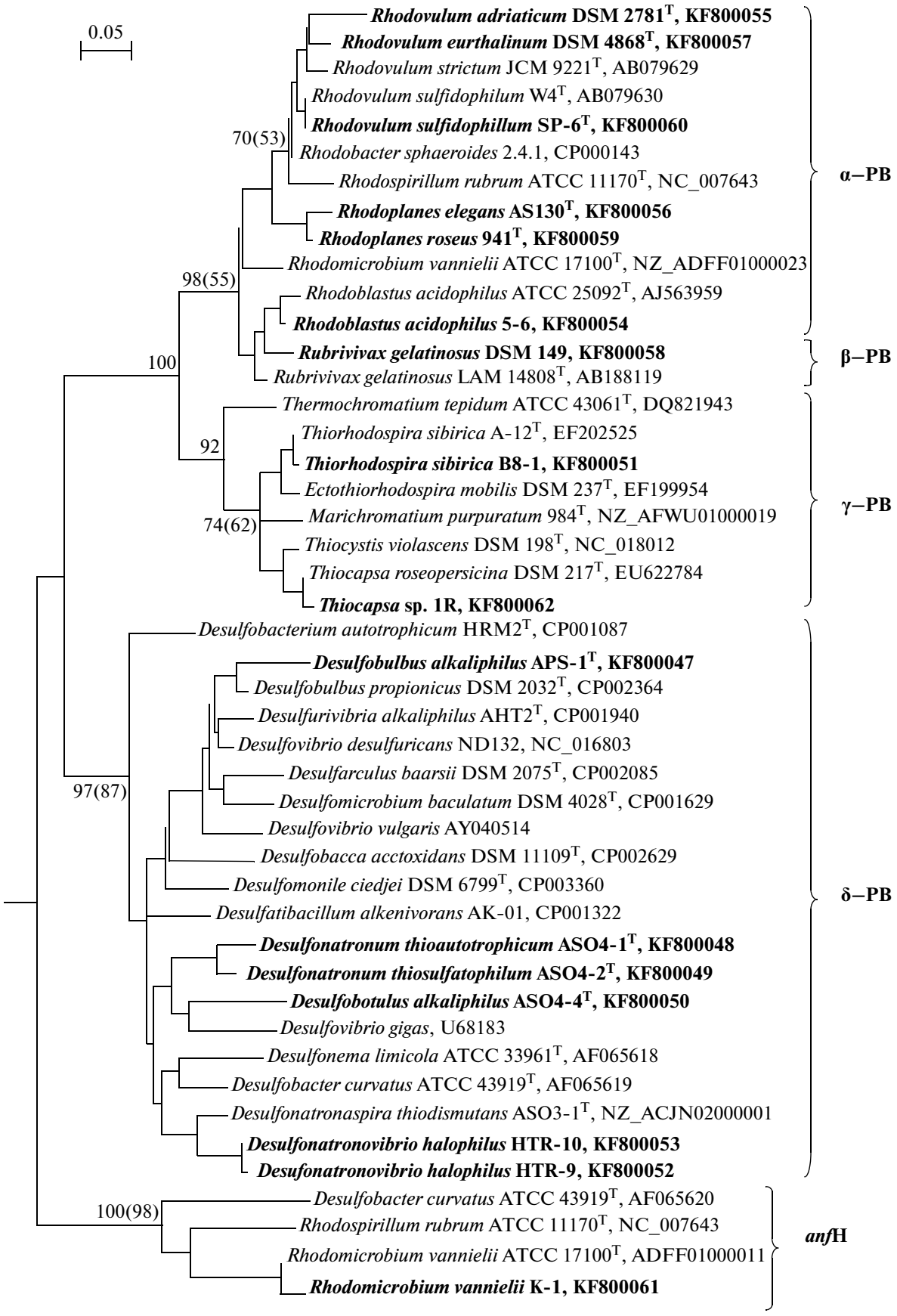
γ–PB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Rhodovulum sulfidophilum W4T, AB079630
Rhodovulum sulfidophillum SP-6T, KF800060
70(53)
Rhodobacter sphaeroides 2.4.1, CP000143
Rhodospirillum rubrum ATCC 11170T, NC_007643
Rhodoplanes elegans AS130T, KF800056
Rhodoplanes roseus 941T, KF800059
Rhodomicrobium vannielii ATCC 17100T, NZ_ADFF01000023
98(55)
Rhodoblastus acidophilus ATCC 25092T, AJ563959
Rhodoblastus acidophilus 5-6, KF800054
Rubrivivax gelatinosus DSM 149, KF800058
100
Rubrivivax gelatinosus LAM 14808T, AB188119
Thermochromatium tepidum ATCC 43061T, DQ821943
Thiorhodospira sibirica A-12T, EF202525
92
Thiorhodospira sibirica B8-1, KF800051
Ectothiorhodospira mobilis DSM 237T, EF199954
Marichromatium purpuratum 984T, NZ_AFWU01000019
74(62)
Thiocystis violascens DSM 198T, NC_018012
Thiocapsa roseopersicina DSM 217T, EU622784
Thiocapsa sp. 1R, KF800062
Desulfobacterium autotrophicum HRM2T, CP001087
Desulfobulbus alkaliphilus APS-1T, KF800047
Desulfobulbus propionicus DSM 2032T, CP002364
Desulfurivibria alkaliphilus AHT2T, CP001940
Desulfovibrio desulfuricans ND132, NC_016803
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97(87)
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Desulfobacca acctoxidans DSM 11109T, CP002629
Desulfomonile ciedjei DSM 6799T, CP003360
Desulfatibacillum alkenivorans AK-01, CP001322
Desulfonatronum thioautotrophicum ASO4-1T, KF800048
Desulfonatronum thiosulfatophilum ASO4-2T, KF800049
Desulfobotulus alkaliphilus ASO4-4T, KF800050
Desulfovibrio gigas, U68183
Desulfonema limicola ATCC 33961T, AF065618
Desulfobacter curvatus ATCC 43919T, AF065619
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Rhodomicrobium vannielii ATCC 17100T, ADFF01000011
Rhodomicrobium vannielii K-1, KF800061
α–PB
β–PB
γ–PB
δ–PB
anfH

**Fig. 2.** Position of the studied strains (set in bold) of diazotrophic proteobacteria in the phylogenetic tree constructed based on analysis of conceptual amino acid translations of *nifH* genes. The tree was constructed with the use of the Neighbor-Joining algorithm. Scale bar shows evolutionary distance corresponding to 5 substitutions per 100 amino acid residues. Numerals at the nodes show the statistical significance of the branching order as determined by bootstrap analysis of 1000 alternative trees (only values higher than 70% are shown). In parentheses, bootstrap values for an analogous tree (not shown) constructed using Maximum Likelihood algorithm are indicated.

ertheless, comparison of the clonal libraries allows semiquantitative extrapolations to be made about the composition of the natural microbial communities in the habitats studied.

The class *Deltaproteobacteria* proved to be ubiquitously represented among all of the NifH libraries studied. The least diverse NifH library, the 14KL library constructed from integrated sediments from the hypersaline Lake Burlinskoe, was represented by the single phylotype 14KL-otu1-20 of diazotrophic sulfate reducers. This phylotype did not exhibit significant similarity either with relevant NifH sequences of sulfate reducers available from GenBank or with newly found NifH sequences of haloalkaliphilic diazotrophic sulfate-reducers (no more than 81.0 and 82.1% identity for nucleotide and amino acid sequences, respectively). The only phylotype that was nearly identical to phylotype 14KL-otu1-20 (97.4 and 98.4% identity for nucleotide and amino acid sequences, respectively) proved to be phylotype 2KL-otu3-6, making up to 26.1% of the clone number in the 2KL library, representing subsurface layer of sediments from the hypersaline Cock Salt Lake. Together, they formed in the tree a separate branch peripherally related to representatives of the order *Desulfovibrionales*, and were evidently affiliated with this order, representing in it a yet-unknown taxon. One more NifH phylotype from the former library, namely 2KL-otu2-6 (26.1% of the number of clones) was closest (83.2 and 96.3% nucleotide and amino acid sequence identity) to the E cluster of clones of uncultured diazotrophic sulfate reducers from the soda Mono Lake [21], and together with this E cluster was part of a cluster of *Desulfuromonadales* representatives, where it was closest to the alkaliphilic iron-reducing soda lake isolate [12] *Geoalkalibacter ferrihydriticus* (84.0% identity for nucleotide sequences and 93.8% identity for amino acid sequences). The remaining four NifH libraries contained a single phylotype of diazotrophic sulfate reducers. These phylotypes were represented by single clones or by minor fractions of clones (1.8–15.0%). These phylotypes were unique for the libraries that they represented; they also formed separate branches in the entire nitrogenase tree that we constructed, exhibiting no pronounced proximity either with relevant sequences available from GenBank or with sequences determined de novo in the present work, although an analogous investigation of the soda Mono Lake did reveal clones of the L cluster close to new haloalkaliphilic sulfate reducers of the genus *Desulfonatronum*. At the same time, two of these minor NifH phylotypes from the libraries derived from sample 6KL (the surface layer of sediments from the soda lake Tanatar-5) and sample 05-3 (integral probe of integrated sediment samples from soda lakes of Bitter system) turned out to be close (92.3 and 78.5% identity for nucleotide sequences and 100 and 95.4% identity for amino acid sequences, respectively) to unidentified deltaproteobacterial sulfate reducers from two other clusters, M and F, detected in Mono Lake. On the whole, the minor NifH phylotypes of sulfate reducers revealed in these four samples were associated with representatives of the orders *Desulfovibrionales* and *Desulfobacterales*, a fact not in contradiction with the results earlier obtained with the use of the molecular marker of sulfate-reducers, the *dsr*B gene [19]. The results of our present study confirm not only the results of that *dsr*B-based study of soda and hypersaline lake of the Kulunda Steppe [19], but also the results of the *nifH*-based studies of Mono Lake. Taken together, these studies strongly suggest the presence in Mono Lake and hypersaline and soda lakes of Kulunda Steppe of so far unknown halo(alkalo)philic diazotrophic sulfate reducers belonging to the class deltaproteobacteria. The ubiquitous presence of the *nifH* genes of sulfate reducers in the samples studied indicate both the considerable contribution of these microorganisms to the NF process in these habitats and the significant role of the sulfate reduction process in these extreme ecosystems.

The next class most represented in the samples studied was *Gammaproteobacteria*. The NiFH phylotypes belonging to it were revealed in five of the six libraries studied. The predominant phylotypes were closely related (81.2–90.2% identity for nucleotide sequences and 94.6–98.5% identity for amino acid sequences); they prevailed in the WN library (integral sample of sediments from alkaline saline Wadi El Natrun lakes) (phylotype WN-otu1-12, 60% of clones) and in the 2KL library (phylotype 2KL-otu1-8, 34.8% of clones). They ranked second in the number of clones (19.3%) in the 05-3 library (phylotype 05-3-otu2-11), or could be represented by a single clone (phylotype 05-3-otu10-1 from the same 05-3 library). These gammaproteobacterial NifH phylotypes formed a coherent cluster in the phylogenetic tree. This cluster was closely related (82.2–90.2% nucleotide sequence identity and 96.3–99.1% amino acid sequence identity) to the C cluster, which had been revealed in an analogous study of the soda Mono Lake. However, this group of phylotypes did not exhibit notable relatedness with cultivated groups of

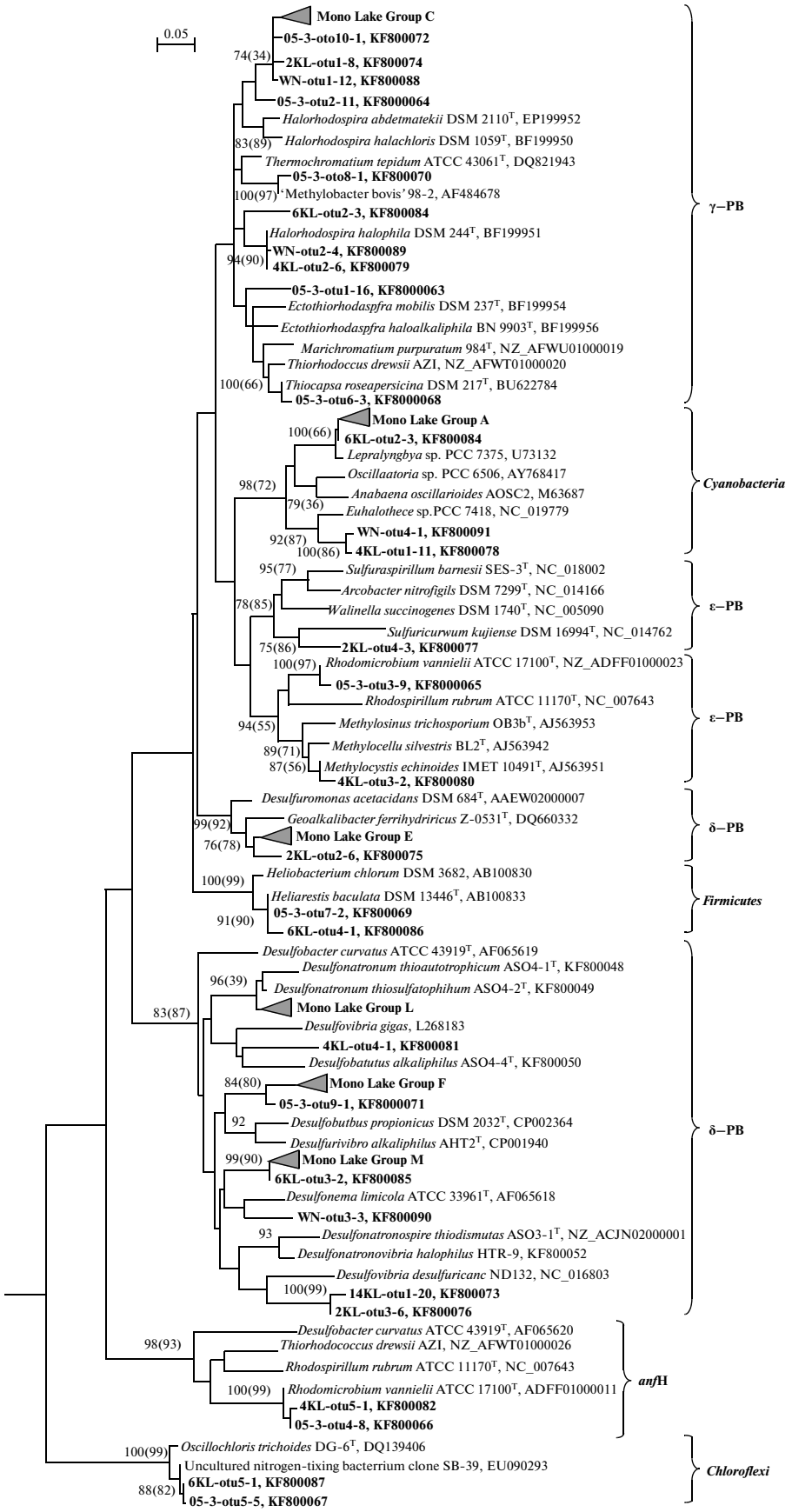
0.05
Mono Lake Group C
05-3-oto10-1, KF800072
74(34)
2KL-otu1-8, KF800074
WN-otu1-12, KF800088
05-3-otu2-11, KF8000064
Halorhodospira abdetmatekii DSM 2110T, EP199952
83(89)
Halorhodospira halachloris DSM 1059T, BF199950
Thermochromatium tepidum ATCC 43061T, DQ821943
05-3-oto8-1, KF800070
100(97)
'Methylobacter bovis' 98-2, AF484678
6KL-otu2-3, KF800084
Halorhodospira halophila DSM 244T, BF199951
94(90)
WN-otu2-4, KF800089
4KL-otu2-6, KF800079
05-3-otu1-16, KF8000063
Ectothiorhodaspfra mobilis DSM 237T, BF199954
Ectothiorhodaspfra haloalkaliphila BN 9903T, BF199956
Marichromatium purpuratum 984T, NZ_AFWU01000019
Thiorhodoccus drewsii AZI, NZ_AFWT01000020
100(66)
Thiocapsa roseapersicina DSM 217T, BU622784
05-3-otu6-3, KF8000068
γ–PB
Mono Lake Group A
100(66)
6KL-otu2-3, KF800084
Lepralyngbya sp. PCC 7375, U73132
Oscillaatoria sp. PCC 6506, AY768417
98(72)
Anabaena oscillarioides AOSC2, M63687
79(36)
Euhalothece sp.PCC 7418, NC_019779
WN-otu4-1, KF800091
92(87)
100(86)
4KL-otu1-11, KF800078
Cyanobacteria
95(77)
Sulfuraspirillum barnesii SES-3T, NC_018002
Arcobacter nitrofigils DSM 7299T, NC_014166
78(85)
Walinella succinogenes DSM 1740T, NC_005090
Sulfuricurwum kujiense DSM 16994T, NC_014762
75(86)
2KL-otu4-3, KF800077
ε–PB
100(97)
Rhodomicrobium vannielii ATCC 17100T, NZ_ADFF01000023
05-3-otu3-9, KF8000065
Rhodospirillum rubrum ATCC 11170T, NC_007643
94(55)
Methylosinus trichosporium OB3bT, AJ563953
89(71)
Methylocellu silvestris BL2T, AJ563942
87(56)
Methylocystis echinoides IMET 10491T, AJ563951
4KL-otu3-2, KF800080
ε–PB
Desulfuromonas acetacidans DSM 684T, AAEW02000007
99(92)
Geoalkalibacter ferrihydriricus Z-0531T, DQ660332
Mono Lake Group E
76(78)
2KL-otu2-6, KF800075
δ–PB
100(99)
Heliobacterium chlorum DSM 3682, AB100830
Heliarestis baculata DSM 13446T, AB100833
05-3-otu7-2, KF800069
91(90)
6KL-otu4-1, KF800086
Firmicutes
Desulfobacter curvatus ATCC 43919T, AF065619
Desulfonatronum thioautotrophicum ASO4-1T, KF800048
96(39)
Desulfonatronum thiosulfatophihum ASO4-2T, KF800049
Mono Lake Group L
83(87)
Desulfovibria gigas, L268183
4KL-otu4-1, KF800081
Desulfobatutus alkaliphilus ASO4-4T, KF800050
84(80)
Mono Lake Group F
05-3-otu9-1, KF8000071
92
Desulfobutbus propionicus DSM 2032T, CP002364
Desulfurivibro alkaliphilus AHT2T, CP001940
99(90)
Mono Lake Group M
6KL-otu3-2, KF800085
Desulfonema limicola ATCC 33961T, AF065618
WN-otu3-3, KF800090
93
Desulfonatronospire thiodismutas ASO3-1T, NZ_ACJN02000001
Desulfonatronovibria halophilus HTR-9, KF800052
Desulfovibria desulfuricanc ND132, NC_016803
100(99)
14KL-otu1-20, KF800073
2KL-otu3-6, KF800076
δ–PB
Desulfobacter curvatus ATCC 43919T, AF065620
98(93)
Thiorhodococcus drewsii AZI, NZ_AFWT01000026
Rhodospirillum rubrum ATCC 11170T, NC_007643
100(99)
Rhodomicrobium vannielii ATCC 17100T, ADFF01000011
4KL-otu5-1, KF800082
05-3-otu4-8, KF800066
anfH
100(99)
Oscillochloris trichoides DG-6T, DQ139406
Uncultured nitrogen-tixing bacterrium clone SB-39, EU090293
6KL-otu5-1, KF800087
88(82)
05-3-otu5-5, KF800067
Chloroflexi

**Fig. 3.** Phylogenetic tree constructed based on analysis of conceptually translated *nifH* genes and showing the positions of phylotypes of uncultured organisms detected in sediments of soda and hypersaline lakes with neutral pH. The names of NifH phylotypes from clone libraries constructed for the samples studied are set in bold; each of these names includes the name of the library, phylotype ordinal number in the library (the order of phylotype numbering follows the decrease in the share of the corresponding clones in the library), and the number of clones represented by the phylotype. Gray triangles comprise phylogenetic clusters of the *nifH* genes detected earlier during the investigation of diazotrophs of the soda Mono Lake [21]. The tree was constructed using the Neighbor-Joining algorithm. Scale bar shows evolutionary distance corresponding to 5 substitutions per 1000 amino acid residues. Numerals at the nodes show the statistical significance of the branching order as determined by bootstrap analysis of 1000 alternative trees (only values higher than 70% are shown). In parentheses, bootstrap values for an analogous tree (not shown) constructed using Maximum Likelihood algorithm are indicated.

gammaproteobacteria; the separate cluster that they formed in the nitrogenase tree was only peripherally related with representatives of purple sulfur bacteria of the order *Chromatiales*. Analogous separate branches related to representatives of the order *Chromatiales* were formed by the dominant phylotype from the 05-3 library (05-3-otu1-16, 28.1% of the number of clones) and a minor phylotype from the 6KL library (6KL-otu2-3, 5.1% of the number of clones). It can be assumed that the phylotypes forming these branches either belong to known taxa whose *nifH* gene sequences are nevertheless still lacking in public databases, or they represent yet unknown taxa of photo- or chemotrophic bacteria of this order. Other NifH phylotypes represented known species and genera of gammaproteobacteria that had been repeatedly isolated from soda lakes by cultivation methods. In the 05-3 library, *Thiocapsa* sp. was revealed (5.3% of the number of clones, 91.0% identity of nucleotide sequences and 99.3% identity of amino acid sequences with *T. roseopersicina* DSM 217$^T$), as was *Methylobacter* sp. (1.7% of the number of clones, 99.3% identity of nucleotide sequences and 98.5% identity of amino sequences with 'M. bovis' 98-2). As for the WN and 4KL libraries (sample of sediment surface from Bitter-1 Lake), we revealed there virtually identical phylotypes WN-otu2-4 and 4KL-otu2-6 (20% and 28.6% of clone numbers, 95.9–98.0% identity for nucleotide sequences and 99.2–100% identity for amino acid sequences); these phylotypes were close (94.8% identity for nucleotide sequences and 99.2% identity for amino acid sequences) to the type strain of the species *Halorhodospira halophila*, which had been for the first time isolated just from the Wadi El Natrun lakes system.

The class *Alphaproteobacteria* was represented in two NifH libraries obtained from soda lake sediments. In the 05-3 library, we revealed phylotypes 05-3-otu4-9 and 05-3-otu5-8 (15.8 and 14.0% of the number of clones), which were virtually identical to the *nifH* and *anfH* genes of *Rm. vannielii* ATCC 17100 (97.8 and 94.1% identity for nucleotide sequences and 99.3 and 99.3% identity for amino acid sequences). Moreover, a single clone of the AnfH phylotype, 4KL-otu5-1 (94.0% identity of nucleotide sequences and 98.4% identity of amino acid sequences with *Rm. vannielii*) was revealed in the 4KL library. The presence of phototrophs of this species is soda lakes was recently confirmed by cultivation methods [26]. Another minor phylotype from the 4KL library, 4KL-otu3-2 (9.5% of the number of clones), represented methanotrophic bacteria of the genus *Methylocystis* (85.7 and 98.5% identity with nucleotide and amino acid sequences of *Mc. echinoides* IMET 10491).

The class *Epsilonproteobacteria* in all of the NifH libraries was represented by a single minor phylotype 2KL-otu4-3 (2KL library, 13% of the number of clones in the 2KL library). This phylotype did not exhibit significant proximity either with known or uncultured representatives of epsilonproteobacteria (no more than 80.4% identity of nucleotide sequences and no more than 86.5% identity of amino acid sequences) and it formed a separate deep branch in the tree, most probably representing a new taxa in this class. It should be mentioned that there are so far no data on the isolation of pure cultures of epsilonproteobacteria from soda lakes; the only data on their possible presence as uncultured autotrophic forms in these habitats obtained in our earlier study were based on the use of a molecular marker, the gene encoding ATP-citrate lyase (*aclB*), the key enzyme of the reductive tricarboxylic acid cycle [16].

Other phylotypes obtained in the NifH libraries belonged to three different lineages of phototrophic bacteria. Most represented were members of the phylum of anoxygenic phototrophic cyanobacteria (*Cyanobacteria*). The phylotype 6KL-otu1-52, which is absolutely dominant in the 6KL library (88.1% of the number of clones), was virtually identical (94.8% identity of nucleotide sequences and 99.1% identity of amino acid sequences) to the A clones obtained in an analogous study of the soda Mono Lake, and, together with these clones, was identified a representative of the genus *Leptolyngbya* (89.5% identity of nucleotide sequences and 99.1% identity of amino acid sequences with *Leptolyngbya* sp. PCC 7375). Representatives of this species are typical filamentous cyanobacteria that inhabit cyanobacterial communities of soda lakes and other saline ecosystems. Two more close phylotypes (98.7% identity of nucleotides sequences and 99.5% amino acid sequences) 4KL-otu1-11, dominating the KL library (52.4% of clone number), and phylotype WN-otu4-1, represented by one clone in the WN library, formed in the tree a separate branch, related (85.8–86.5% identity for nucleotide sequences and 93.0–93.7% identity for amino acid sequences) to

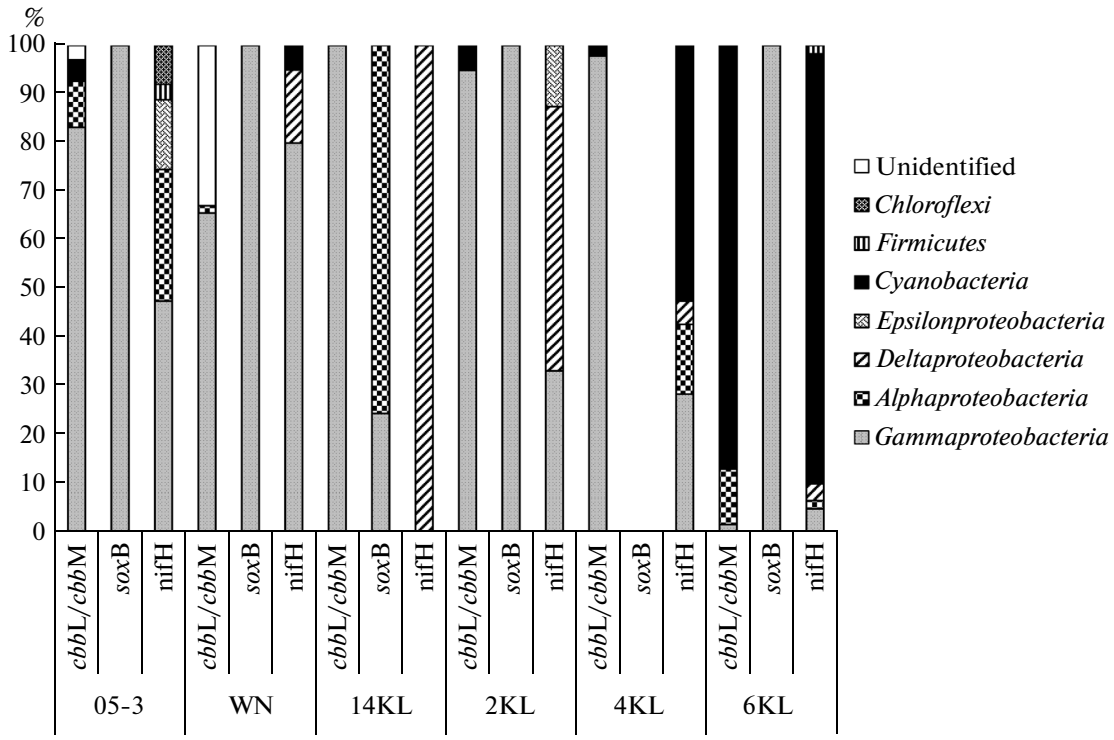


**Fig. 4.** Phylogenetic distribution of clones in the libraries derived from sediment samples of soda and hypersaline lakes with neutral pH with the use of the *cbb*L*/cbb*M, *sox*B and *nifH* genes as molecular markers. The "Unidentified" label denotes clones whose phylogenetic identification failed to be identified.

representatives of halophilic unicellular cyanobacteria from the phylogenetic group *Euhalothece* sensu Garcia-Pichel et al. [27]. Given that the NifH phylotypes that we detected were found in soda lake samples, it is highly possible that they may represent an extremely natronophilic species of this group of cyanobacteria: *E. natronophila* [28].

In the 05-3 and 6KL NifH libraries from soda lakes, we detected virtually identical phylotypes (98.5% identity for nucleotide sequences and 98.5% identity for amino acid sequences): a minor phylotype 05-3-otu7-2 (3.5% the number of clones) and a single-clone phylotype 6KL-otu4-1. These phylotypes belonged to the photoheterotrophic heliobacteria of the genus *Heliorestis* (98.2–99.4% identity of nucleotide sequences and 98.2–100% identity of amino acid sequences with *H. baculatus* DSM 13446). In the same two libraries, we revealed completely identical phylotypes (100% identity of nucleotide and amino acid sequences): a minor phylotype 05-3-otu5-5 (8.8% of the number of clones) and phylotype 6KL-otu5-1, represented by a single clone. These phylotype(s) belonged to the final group of phototrophs found in this work, namely, to green nonsulfur filamentous bacteria of the genus *Oscillochloris* (90.9–91.0% identity of nucleotide sequences and 97.0–97.8% identity of amino acid sequences with *O. trichoides* DG-6). Representatives of these groups of phototrophic anoxygenic bacteria (as well as representatives of purple nonsulfur bacteria of the genus *Rhodomicrobium*) have been repeatedly isolated from soda lakes in the past; however, those soda lakes had much lower mineralization levels (of up to 35 g/L [26]) than the soda lakes studied in the present work. Therefore, it can be assumed that at higher mineralization levels these organisms resided in an inactive state, being activated upon changes in the environmental conditions (seasonal and/or local dilution of the brine in the periods of flood or heavy precipitation).

Previously, cultivation and molecular methods [4, 8–11, 20] showed the presence in the soda lake sediments of firmicutes (clostridia and bacilli) and methanogenic archaea, i.e., of microorganisms, many of which are capable of NF. Nevertheless, our present analysis failed to reveal NifH phylotypes of these organisms in the libraries that we constructed. The reasons may be due both to insufficient universality of the primer system we used and to local distinctions in the microbial communities of the studied natural habitats. It should also be noted that analogous discrepancies were noted in studies of the water column of the soda Mono Lake: while data from 16S rRNA gene analysis in the anaerobic near-bottom layer revealed considerable diversity of firmicutes [29], no NifH phylotypes of these organism have been detected [21].

In spite of the fact that phylotypes of diazotrophic bacteria were revealed in all of the soda and saline lake samples that we studied, the general diversity of NifH

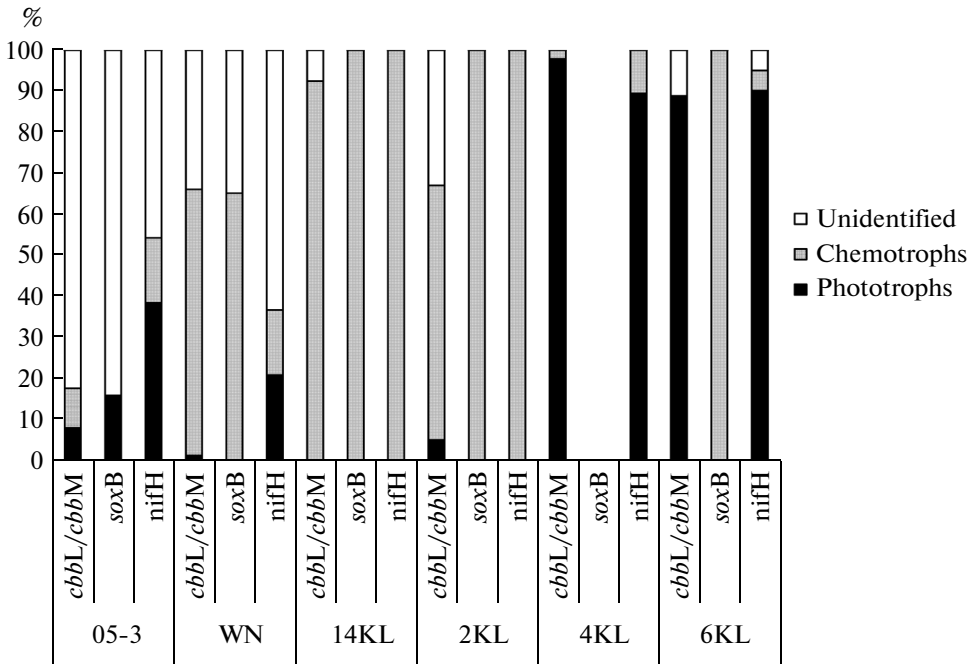


**Fig. 5.** Ratios of clones representing photo- and chemotrophic bacteria in *cbb*L/*cbb*M, *sox*B, and *nifH* gene libraries derived from sediment samples from soda and hypersaline lakes with neutral pH. The "Unidentified" label denotes clones whose type of metabolism could not be supposed.

phylotypes proved to be low, varying from 0.05 to 0.24 phylotype/clone, which is close to the value (0.19) recorded in the soda Mono Lake. This fact may be due to the steadily extreme environmental conditions, which provide advantage to one or two best adapted organisms. This, however, does not exclude changes in the dominant forms upon seasonal and/or local variations in the environmental conditions, especially important of which are decreases in salinity. A second possible factor that may regulate the development of diazotrophs in soda lake sediments is seasonal variations is the concentration of bound nitrogen (ammonia in particular). Our unpublished data show high (up to 1 mM) ammonia concentrations in the pore waters of the soda lakes of the Bitter and Tanatar systems in the summer period; this should suppress the activity of diazotrophs.

**Comparative analysis of bacterial communities of saline and soda lake sediments with the use of various functional molecular markers.** Samples investigated in this work had earlier been used for the detection of other functional molecular markers, namely, the key genes of autotrophy (*cbb*L/*cbb*M) [15–17] and sulfur oxidation (*sox*B) [18], thus giving us an opportunity to compare the results of the present study with the results of those studies.

Comparison of the results of application of various functional genes as molecular markers for the analysis of microbial communities in sediments of saline and soda lakes showed the predominance of gammaproteobacteria in the constructed libraries; they were present in all samples and could be revealed with the use of all of the markers employed (Fig. 4). The only exception was the NifH library derived from sample 14KL. The lack of gammaproteobacterial phylotypes in this NifH library could be due to the nearly complete dominance in this sample (evidenced by analyses of CbbL/CbbM and SoxB libraries) of the phylotypes of autotrophic sulfur-oxidizing gammaproteobacteria *Thiohalorhabdus*, which are not diazotrophs. As for the phyla other than the nearly ubiquitous *Gammaproteobacteria*, their detection in the constructed libraries varied, as it was dependent on the sample and the marker gene used.

When the taxonomic composition of the identified phylotypes was analyzed, the fact that attracted attention first of all was that in no sample was a phylotype of an organism revealed whose genome unambiguously combined all of the three analyzed marker genes. The only organism for which such a combination looks quite possible is *H. halophila*, found as a dominant autotrophic phylotype and second-ranking diazotrophic phylotype in the 4KL sample. The failure to amplify the *sox*B of this sulfur-oxidizing organism in the same sample seems to be due exclusively to the insufficient universality of the applied primer system [18]. It is possible that some of the other samples also contained autotrophic nitrogen-fixing sulfur-oxidizing bacteria. Particularly, in sample 05-3 we revealed, as a minor component, a SoxB phylotype belonging to the genus *Halochromatium*. Several of the CbbL and NifH phylotypes probably are affiliated to the family

*Chromatiaceae*; however, these phylotypes cannot so far be precisely identified because public databases so far lack *cbbL* and *nifH* gene sequences of representatives of the genus *Halochromatium*. The fraction of unidentified phylotypes associated with the order *Chromatiales* in sample 05-3 was, on the whole, rather large, and this allows us also to assume the presence among them of additional phylotypes of autotrophic nitrogen-fixing sulfur-oxidizing bacteria. These phylotypes could represent either unknown taxa of this order or taxa whose relevant marker genes are not yet represented in the databases.

Comparison of the results for three different functional markers demonstrated considerable overlapping of the identified phylotypes prevalent in the clonal libraries; at the same time, the composition of the minor components with the same combinations of marker genes showed notable variations. For example, according to the analyses of the *cbb*L/*cbb*M and *sox*B genes, *Thioalkalivibrio* phylotypes prevailed in the WN library, *Thiohalorhabdus* phylotypes predominated in the 14KL library, and *Halothiobacillus* phylotypes, in the 2KL library. Analogously, according to the analyses of the *cbb*L/*cbb*M and *nifH* genes, *Halorhodospira* phylotypes prevailed in the 4KL library; cyanobacterial phylotypes, in the 6KL library. The compositions of the libraries derived from sample 05-3 were the most diverse in terms of all of the three marker genes used; however, it proved impossible to determine the ratio of the predominant components of this microbial community because of the high content of unidentified phylotypes.

Comparative analysis of the constructed libraries also allowed the ratios between identified photo- and chemotrophic components to be determined in the microbial communities studied (Fig. 5). In hypersaline lakes with neutral pH, we revealed only minimal presence of phototrophs: in the integrated sediment of sample 14KL, no known phototrophic taxa were found. In the surface sediment sample 2KL, cyanobacterial components were found, but only in the CbbL library and only as a minor component. Phototrophs were more diverse in sediment samples from soda lakes, but their quantitative ratios were different in surface samples and integrated samples. The lowest number of phototrophs was recorded in integrated sediments from sample WN: only in the NifH library were phylotypes of *Halorhodospira* and cyanobacteria revealed as minor components, in spite of the fact that phototrophs—at least anoxygenic phototrophs—are easily isolated from Wadi El Natrun lakes. In the integrated sediments of sample 05-3, phototrophs were also minor components of the community; however, they were more diverse. *Ectothiorhodosinus, Rhodovulum*, and cyanobacteria were represented in the CbbL library; *Halochromatium* was represented in the SoxB library; and *Rhodomicrobium*, *Thiocapsa*, *Oscillochloris*, and *Heliorestis* were present in the NifH library. In samples of surface sediments of soda lakes, phototrophs prevailed: in CbbL and NifH libraries of sample 4KL, *Halorhodospira* and cyanobacteria predominated, whereas in analogous libraries of sample 6KL, cyanobacteria were dominant. Additionally, in the NifH library of sample 6KL, phylotypes of *Rhodomicrobium, Oscillochloris*, and *Heliorestis* were present as minor components.

To conclude, comparative analysis of the results obtained with the three functional molecular markers allowed us to considerably expand the knowledge of the natural communities of the sediments of hypersaline lakes in respect to the diversity of community members with various types of metabolisms (Table 3). As distinct from the traditional analysis that employs only 16S rRNA gene as a marker, the complex analysis with the use of several marker genes of key enzymes of various metabolic processes makes it possible to reveal minor components of extremophilic microbial communities that may nevertheless play an important role in the community functioning. In particular, the analysis of 16S rRNA genes earlier conducted for sediments of Kulunda Steppe soda lakes failed to reveal either anoxygenic phototrophs or representatives of the genus *Thioalkalivibrio*, specific to soda lakes [4]. Thus, the complex analysis with the use of several marker genes allows more adequate and profound planning of further experiments on the isolation of pure cultures of unknown taxa of halo(alkalo)philic bacteria. As for the presence and activity of diazotrophic cyanobacteria in soda and hypersaline lakes and their detailed taxonomic composition, these questions remain open and await both field measurements of activities and studies with available pure cultures and environmental samples.

## ACKNOWLEDGMENTS

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